THE GENETIC ROLE OF STAPHYLOCOCCUS AND STREPTOCOCCUS PHAGES IN C. diphtheriae TOXINOGENESIS

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Studies of the transformation of nontoxigenic diphtheria corynebacteria into toxigenic variants after association with the lytic diphtheria phage B showed for the first time that phage is the genetic determinant which controls toxinogenesis (13). Since then it was established that although toxin production is under the genetic control of a specific phage (6, 14, 15), the conversion of toxigenicity is possible only in some C. diphtheriae strains (8, 16, 19). It was shown that the conditions necessary for toxin production by diphtheria corynebacteria also lead to the multiplication of diphtheria phage (7). This brought up the idea that the synthesis of phage possibly is one of the conditions necessary for C. diphtheriae toxinogenesis. In further investigations it was shown that toxin formation by diphtheria corynebacteria occurs in two stages: I is linked with the dynamics of the phage growth and can be inhibited by several antibiotics, II takes place after lysis of the diphtheria corynebacteria, characterized by an increase in the amount of toxin, and can be blocked by the addition of iron (9).

Investigations directed toward the study of the possible role of interspecies genetic mechanisms in a natural mutation of C. diphtheriae under conditions of microbial association with staphylococci and streptococci, among which there are many lysogenic strains takes on particular significance (4, 5, 20, 21). Lysogenesis was also established in Corynebacteria diphtheriae (12, 17, 22). During natural lysogenesis the conditions may arise for the transformation of nontoxigenic strains of C. diphtheriae into toxigenic not only from the effect of some specific phages but also as a result of interaction between staphylo- and streptophages of lysogenic cultures of these microbes. Rumanian investigators (10, 11) have reported on the possibility of the conversion of nontoxigenic strains of C. diphtheriae into toxigenic variants in vitro under the influence of staphylococcic sphages. Earlier, we observed in animals the transformation of a nontoxigenic strain of C. diphtheriae into its toxigenic variant during association both with staphylococci and with streptococci (2).

The purpose of the present work was an investigation of the possible genetic role of staphylo- and streptophages in C. diphtheriae toxigenesis.

EXPERIMENTAL METHODS

Nontoxigenic nonlysogenic C. diphtheriae Freeman 411, 444, 770, 1174, 1180 (mitis type), 304 (gravis type, Cophenhagen) and the control toxigenic strain gravis type No. 3148 (Chuk) were used in the experiments.

Studies of the effect of heterogenous phages on the toxinogenesis of C. diphtheriae were carried out with an international collection of 22 standard staphylococcus phages and with four streptococcus phages, 9, 42, 60 (moderate) and CA1 (virulent) kindly given by A. K. Totolyan. Fermentative activity was investigated in media with glucose, maltose, mannitol, lactose, sucrose, starch, and glycerine. Biological type was determined from the ability to ferment starch. Virulence of the diphtheria corynebacteria was studied in guinea pigs by the Eagleton-Baxter method. Toxigenic properties were investigated in vitro by Ilek's method on phosphate-peptone agar and in vivo in guinea pigs. Experiments with staphylo-and streptophages were carried out on 1.5% heart-brain agar (18), first inoculated with a 1½ hour C. diphtheriae culture in Pope-Lingood broth. The Petri dishes were dried at 37° and then drops of the appropriate phages placed on the surface with a loop. A drop of 0.85% NaCl solution served as a control. After drying the moisture at room temperature the plates were incubated for 16-18 h at 37°. On the following day either

TABLE 1. Frequency and Stability of Toxin Production by C. diphtheriae Variants During Interaction with Staphylococcus and Streptococcus Phages

Diphtheria variant	Number of cultures studied	Number of toxigenic subcultures		Toxigenicity of subcultures			
				retained		lost	
		absolute	%	absolute	%	absolute	%
411/9	20	7	35	7	35	0	0
411/CA1	20	8	40	2	10	6	30
411/54	20	6	30	0	0	6	30
1174/71	20	9	45	0	0	9	45
1174/9	20	16	80	0	0	16	80
770/6	20	4	20	0	0	4	20
770/52A	20	2	10	0	0	2	10
770/53	20	2	10	0	0	2	10
770/544	20	2	10	0	0	2	10
770/545	20	5	25	0	0	5	25
770/546	20	1	5	0	0	1	5
444/52A	20	1	5	0	0	1	5
444/53	20	1	5	0	0	1	5
304/53	20	1	5	0	0	1	5
304/54	20	1	5	0	0	1	5
Total	300	66	22	9	13,64	57	86,36

secondary colonies (not less than 10) were removed if lysis of C. diphtheriae was observed, or bacteria were removed with a loop from several areas where phage had been applied. Later, the cultures removed daily were grown and 20 subcultures of each of the investigated variants examined as to morphological, cultural, fermentative, virulent and toxigenic characteristics.

RESULTS

As seen from Table 1, of 300 cultures studied 66 (22%) produced toxin.

Not all strains of the diphtheria corynebacteria studied were transformed to the same degree into toxigenic forms. It occurred most frequently in strain No. 1174 under the influence of streptophage 9 (80% of the colonies studied) and staphylophage 71 (45%), and also in strain No. 411 in which transformation into the toxigenic variant was noted with streptophage 9b (35%, 7 subcultures) and CA1 (40%, 8 subcultures). In the remaining toxigenic variants of diphtheria corynebacteria 779, 444, 304 toxigenicity varied from 5-25% of the colonies examined. Diphtheria cultures 770 and 444 were lyzed by several staphylophages, but the variants isolated had unstable toxigenic properties (see Table 1). We did not once note visible lysis as a result of streptophages on the experimental strains of diphtheria corynebacteria. In the place where phage had been applied a barely noticeable trace of the drop was observed which was difficult to distinguish from the drop in the control. In this case we isolated 9 culture variants with stable toxigenicity (see Table 1). It is probable that lysis of the bacteria is not obligatory for the formation of toxigenic variants (in the case with streptophages), as is may be with diphtheria phages (7), but that contact with the phage is sufficient.

The stability of the toxigenity of the C. diptheriae variants obtained deserves particular attention. As seen from Table 1, only 9 variants (13.64%) which arose from the effect of streptococcus phages 9 and CA1, always retained the acquired toxigenic properties during the whole period of investigation, the remaining subcultures lost this characteristic after 3-6-10 transfers.

In Table 2 data is presented on toxin production by stable toxigenic C. diphtheriae variants. Fermentative activity is characterized as a convenience in relation to glucose, sucrose and starch.

To show specific toxin production, stable toxigenic variants and a control toxigenic strain (No. 3148) were grown in Martin broth at pH 8.0 for 10-12 days. Introduction of 0.1 ml of culture filtrate in a dilution of 10^{-4} induced the formation of expressed necrosis (see Table 2). In 2-3 days the animals died from a typical diphtheria intoxication; control animals immunized with anti-diphtheria serum remained alive. Then the experiments were repeated with another series of toxin obtained from the same cultures, and also with subcutaneous injection of guinea pigs with 6 million cells of the toxigenic variants and the control strain we noted death of the animals after 1-3 days. Preliminary introduction of antidiphtheria serum in these cases prevented death.

Three series of toxins were examined for flocculation reaction with standard anti-diptheria serum; positive results were not obtained once, while a sample determination of the Dlm of toxigenic variants 411/9—second subculture, 411/CA1—second subculture showed a two-fold increase in Dlm (0.025 ml) over the control toxigenic strain No. 3148 (0.05 ml).

The experiments show that with toxin production by C. diphtheriae during interaction of the cells and phage there can occur lysogenic conversion caused not only by diphtheria and staphylococcus phages (8, 10, 11, 17). In our experiments with ultraviolet irradiation of 9 stable toxigenic variants of 411/9 and 411/CA1 we were not able to determine whether these variants remained lysogenic after contact with streptophages 9 and CA1 (virulent). It is possible that the negative result is caused by the limited number of indicator strains used in the experiment, and

TABLE 2. Toxin Production by Stable Toxigenic C. diphtheriae Variants Obtained Under the Influence of Staphylo- and Streptophages

Strain No.	Fermentative activity			Biological type	Virulence in vivo (necrotic area in mm)	Toxigenicity	
	glucose	sucrose	starch			in vitro	in vivo (necrotic area in mm)
411 Parent	K	—	—	Mitis		—	—
411/9 Subculture 1 . . .	K	—	K	Gravis	20×25	+	5×5
411/9 » 2 . . .	K	—	K	»	18×22	+	15×15
411/9 » 3 . . .	K	—	K	»	15×15	+	5×5
411/9 » 6 . . .	K	—	K	»	10×12	+	15×15
411/9 » 12 . . .	K	—	K	»	15×15	+	8×10
411/9 » 15 . . .	K	—	K	»	20×20	+	15×15
411/9 » 18 . . .	K	—	K	»	20×20	+	5×5
411/CA1 » 2 . . .	K	—	K	»	20×20	+	15×15
411/CA1 » 5 . . .	K	—	K	»	18×18	+	5×5
3148 Control	K	—	K	»	20×20	+	10×10

Symbols: K—fermentation with acid production; —negative result; + positive reaction.

perhaps the conversion of the nontoxigenic strain No. 411 into its toxigenic variant occured in a type of transduction, caused by streptophages which carry an interspecies character. Proof of this hypothesis requires conducting broad experiments in this direction, since the participation of various genetic mechanisms in C. diphtheriae toxin production continues to remain uninvestigated. The wide distribution in nature of staphylococci and streptococci and the large number of lysogenic strains among these microbes found in symbiosis with lysogenic diphtheria corynebacteria in the human nose and throat can create conditions for the mutual exchange of genetic determinants which control toxinogenesis in C. diphtheriae.

Thus, in our material, transmission of about genetic information toxinogenesis to the diphtheria corynebacteria by staphylococcic and streptococcic phages is observed in 22% of the cases. Stable toxin production is noted during interaction with streptophages (13.64% with respect to 66 toxigenic variants) and has a temperary nature in the variants arising from the effect of staphylophages. The results of the investigation show that, along with other factors, staphylococcus and streptococcus phages play an important role in the natural evolution of C. diphtheriae toxinogenesis.

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